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Serial exposure to ethanol drinking and methamphetamine enhances glutamate excitotoxicity.

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Abstract

A significant comorbidity exists between alcohol and methamphetamine (Meth) abuse but the neurochemical consequences of this co-abuse are unknown. Alcohol and Meth independently and differentially affect glutamatergic transmission but the unique effects of their serial exposure on glutamate signaling in mediating damage to dopamine neurons are unknown. Sprague Dawley rats had intermittent voluntary access to 10% ethanol (EtOH) every other day and water over 28 days and were then administered a binge injection regimen of Meth or saline. EtOH drinking decreased the glutamate aspartate transporter (GLAST) and increased basal extracellular concentrations of glutamate within the striatum when measured after the last day of drinking. Ceftriaxone is known to increase the expression and/or activity of glutamate transporters in the brain and prevented both the decreases in GLAST and the increases in basal extracellular glutamate when administered during EtOH drinking. EtOH drinking also exacerbated the acute increases in extracellular glutamate observed upon Meth exposure, the subsequent increases in spectrin proteolysis, and the long-term decreases in dopamine content in the striatum, all of which were

attenuated by ceftriaxone administration during EtOH drinking only. These results implicate EtOH-induced increases in extracellular glutamate and corresponding decreases in glutamate uptake as mechanisms that contribute to the vulnerability produced by EtOH drinking and the unique neurotoxicity observed after serial exposure to Meth that is not observed with either drug alone.

Keywords

1. Alcohol
2. Methamphetamine
3. Glutamate
4. Excitotoxicity
5. Dopamine
6. GLAST

Abbreviations

1. aCSF……Artificial cerebrospinal fluid
2. CamKII…….Calcium-calmodulin-dependent protein kinase II
3. CEF……...Ceftriaxone
4. COX-2……..Cyclooxygenase-2
5. EAAT1/2……..……Excitatory amino acid transporter ½
6. EtOH……..Ethanol
7. GLAST……..Glutamate aspartate transporter

8. GLT-1……Glutamate transporter 1
9. LPS……Lipopolysaccharide
10. Meth……...Methamphetamine
11. NMDA…… N-methyl-D-aspartate
12. OPA…….. *o*-pthaldialdehyde
13. RRID……..Research Resource Identifier (see scicrunch.org)
14. SBP……Spectrin breakdown product

Introduction

Alcohol and methamphetamine (Meth) are commonly co-abused and act on similar neurotransmitter systems. Abuse of these drugs is a significant problem highlighted by a recent study from the National Epidemiologic Survey on Alcohol and Related Conditions reporting that alcohol use, high-risk drinking, and DSM V-diagnosed alcohol use disorders have all increased in the United States (Grant *et al.* 2017). Furthermore, abuse of alcohol and Meth is a prevalent comorbidity amongst Meth users (Stinson *et al.* 2005; Bujarski *et al.* 2014; Leslie *et al.* 2017), but the causes and consequences of their co-abuse have only been recently examined (Fultz *et al.* 2017) and remain undefined.

A mechanism underlying the toxic effects of alcohol includes inflammation derived from the gastrointestinal (GI) tract, liver, and brain (Massey & Arteel 2012; Bishehsari *et al.* 2017; Leclercq *et al.* 2017; Vallés *et al.* 2004; Wang *et al.* 2010) whereas the neurotoxic effects of Meth are mediated by neuroinflammation and excitotoxicity (Mark *et al.* 2004; Battaglia *et al.* 2002; Stephans & Yamamoto 1994; Thomas & Kuhn 2005). Enhanced dopamine toxicity has been observed after the serial exposure to alcohol and Meth in the striatum that may be mediated by neuroinflammation via increases in cyclooxygenase-2 (COX-2) from alcohol

drinking (Blaker & Yamamoto, 2018) but this requires further investigation. Although neuroinflammation can promote glutamate-mediated excitotoxicity that in turn, contributes to neuronal injury (Lawrence *et al.* 1998; Stroemer & Rothwell 1998; Allan *et al.* 2000; Ye *et al.* 2013), the contribution of glutamate signaling to enhanced dopamine neurotoxicity after the serial exposure to alcohol and Meth is unknown.

A possible mechanism by which inflammation promotes glutamate-mediated excitotoxicity is through decreased glutamate uptake. Glutamate uptake occurs through glutamate transporter-1 (GLT-1, also known as excitatory amino acid transporter-2/EAAT2) on presynaptic terminals and astrocytes, and the glutamate aspartate transporter (GLAST, also known as excitatory amino acid transporter-1/EAAT1), located primarily on astrocytes (Chen et al., 2004; Roberts *et al.* 2014). Reductions in glutamate uptake are observed after chronic alcohol abuse which likely occurs through a decrease in transporter-mediated uptake (Kryger & Wilce 2010; Flatscher-Bader & Wilce 2008) or a functional reversal of transporter activity resulting in the increased availability of extracellular glutamate (Alhaddad *et al.* 2014) and activation of postsynaptic receptors to produce excitotoxicity.

Excitotoxicity is also a consequence of increases in glutamate release. Meth causes acute increases in extracellular glutamate within the striatum (Mark *et al.* 2004; Nash & Yamamoto 1992) to activate glutamate receptors and increase proteolysis of the cytoskeletal protein spectrin (Siman and Noszek,1988) that comprises nerve terminals in the striatum. Specifically, spectrin proteolysis from Meth-induced excitotoxicity contributes to dopamine terminal degradation in the striatum (Siman & Noszek 1988; Halpin *et al.* 2014; Staszewski & Yamamoto 2006; Tata & Yamamoto 2007). Spectrin may be cleaved by caspase-3, a calcium-dependent protein that is activated by increases in calcium influx, into a breakdown product with a molecular weight of 120 kDa (Pike *et al.* 2001). Proteolysis by other calcium-

activated proteases results in breakdown products of different molecular weights. In rodent models of striatal dopamine loss after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) injections, the 120 kDa caspase-3 mediated spectrin breakdown products have been detected in the striatum prior to dopamine terminal loss (Grant *et al.* 2009) implicating it as a key contributor to dopaminergic damage. However, It remains to be determined if spectrin proteolysis by caspase-3 occurs after the serial exposure to alcohol and Meth in a manner associated with increases in glutamate release and a reduction in glutamate transporters.

The β-lactam antibiotic ceftriaxone (CEF) has been shown to be neuroprotective by increasing the expression and/or activity of the glutamate transporters in the brain (Rothstein *et al.* 2005; Wei *et al.* 2012). It protects against excitotoxicity in rodent models of drug abuse, stroke, traumatic brain injury, Parkinson's disease, and hypoxia (Goodrich *et al.* 2013; Hameed *et al.* 2018; Cui *et al.* 2014; Barr *et al.* 2015; Lipski *et al.* 2007; Leung *et al.* 2012; Jagadapillai *et al.* 2014). In addition, CEF has anti-inflammatory effects (Wei *et al.* 2012) but it remains to be determined if it protects against the neurotoxic effects associated with the comorbidity of alcohol and Meth.

The current study expands upon previous findings by Blaker and Yamamoto (2018) reporting a role for inflammation in the enhanced dopamine toxicity observed after the serial exposure to EtOH+Meth. Because inflammation may promote glutamate-mediated excitotoxicity and contribute to neuronal damage after either drug alone, the current study investigated the role of glutamate signaling in mediating enhanced dopamine depletions after EtOH+Meth. We tested the hypothesis that prior alcohol drinking decreases glutamate transporters and increases basal extracellular glutamate in a manner that exacerbates the acute increases in extracellular glutamate and excitotoxicity produced by Meth, as evidenced by breakdown of

the cytoskeletal protein spectrin and the associated neurotoxicity defined by the loss of dopamine content. Furthermore, we predicted that the administration of the β-lactam antibiotic, CEF during alcohol drinking will prevent the excitotoxicity and the enhanced depletions of dopamine observed after the serial exposure to alcohol and Meth.

Materials and Methods

Animals

Male Sprague Dawley rats (RRID:MGI:5651135**;** 175-199g; Envigo, Indianapolis, IN) were allowed one week to acclimate to the animal colony at Indiana University School of Medicine before beginning experiments. Much of the previous work related to these experiments have used male rat models and therefore we used all males throughout experiments to expand upon previous findings. Any identification of sex differences between males and females would require future studies. All experiments were carried out and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Indiana University Institutional Animal Care and Use Committee (Protocol #11042). This study was not pre-registered and no randomization was used to allocate animals to treatment groups.

Drug Treatment

The graphical timeline for all experiments can be viewed in Figure 1. After the initial acclimation period, rats were individually housed in order to accurately measure 10% ethanol (EtOH) intake per rat. As described in Blaker and Yamamoto (2018), rats were allowed access to two bottles of water or access to one bottle of 10% EtOH and a second bottle of water every other day for a total period of 28 days, and *ad libitum* access to food. On the days without EtOH access, a second water bottle was placed on the cage permitting

access to two bottles at all times. Additionally, the placement of the EtOH bottle was altered upon each exposure as to prevent side bias.

One day after the last day of EtOH drinking, rats were exposed to a binge injection regimen consisting of a total of 4 Meth (10 mg/kg) or saline injections, each injection 2 hours apart in the morning. (+) Methamphetamine-hydrochloride (Sigma, St. Louis, MO, cat #M-8750) was dissolved in 0.9% saline and control rats received injections of 0.9% saline (1 mL/kg) at the same time. This particular drug administration paradigm leads to comparable dopamine deficits observed in human brains (Callaghan *et al.* 2012; Volkow *et al.* 2001; McCann *et al.* 1998). Additionally, the investigator-administered model permits control over the time course of injections and specific doses in order to clearly interpret the discrete pharmacological effects of Meth that are not feasible with a Meth self-administration paradigm.

Body temperatures were measured every 30 minutes throughout the binge Meth regimen via subcutaneous temperature transponders (IPTT-300 transponder, BMDS) which were implanted one day prior to the binge Meth regimen. All rats received temperature transponders, regardless of Meth or saline treatment.

A subset of rats (n=76) received injections of the β-lactam antibiotic ceftriaxone (Sigma cat #1098184; 200 mg/kg, ip) as described in Hu *et al.* (2015) once daily on days without EtOH access, to counter any decreases in glutamate transporters due to EtOH. Control rats received 0.9% saline injections (1 mL/kg).

Western Blotting

Rats were rapidly decapitated without anesthetic 24 hr after the last day of EtOH access for GLAST and GLT-1 quantification, or 7 days after Meth for spectrin breakdown product (SBP) quantification. Striata were dissected, immediately frozen on dry ice, and homogenized in RIPA buffer for GLAST and GLT-1. Striatal tissue from rats euthanized at 7 days was dissected around the probe after microdialysis experiments and homogenized in a buffer comprised of 10 mM Tris, 10 mM EGTA, 250 mM sucrose, and HALT protease inhibitor cocktail (Thermo Scientific) as described in Halpin *et al.* (2014) for SBP analysis. Protein was quantified via Bradford assay. All samples for GLAST and GLT-1 quantification were reduced with 500 mM DTT (Fisher cat #PR-V3151) whereas those for spectrin were not. All samples for GLAST, GLT-1, and spectrin were diluted in Novex 4X LDS sample buffer (Invitrogen cat #NP0007) and 20 μg or 30 μg protein was loaded into each lane of 4-12% Bis-Tris gels (Invitrogen cat #NP0335) for GLAST/GLT-1 or spectrin, respectively, for SDS-PAGE gel electrophoresis at 150V for 90 minutes. Proteins were transferred onto PVDF membrane for 2 hrs at 28V and then placed in 5% non-fat milk blocking buffer for 1 hr at room temperature. Membranes were incubated with primary antibody for the GLAST homolog EAAT1 (raised in rabbit; 1:1000, RRID:AB_304334; Abcam cat #ab416), guinea pig anti-GLT-1 (1:1000, RRID:AB_90949, Millipore cat #MAB1783), or mouse anti-non-erythroid α-spectrin (RRID:AB_94295, Millipore cat #MAB1622) for 18 hrs at 4°C, washed with TBS containing 0.5% Tween, and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, 1:2500, RRID:AB_631748, Santa Cruz cat #SC2054; goat anti-guinea pig IgG, 1:3000, RRID:AB_650492, Santa Cruz cat #sc-2438; or anti-mouse IgG, 1:2500, RRID:AB_2687626, Santa Cruz cat #SC516102) for 1 hr at room temperature. Membranes were washed again with 0.5% TBS-T, incubated with HyGLO-enhanced chemiluminescence and imaged with a FujiFilm LAS-4000 camera. Band density was acquired with Multi-Gauge V3.1 software and normalized against β-actin (1:3000,

RRID:AB_2223041, Millipore cat #MAB1501) or α-tubulin (1:2000, RRID:AB_477582, Sigma cat #T6074).

In Vivo Microdialysis

Microdialysis probes were constructed using 26 gauge stainless steel hypodermic tubing (Small Parts), PE 20 tubing (Becton Dickinson), silica tubing (150 μm OD; Polymicro Technologies), 4 mm length hollow fiber microdialysis membrane (13kD molecular weight cutoff; 216 μm OD; Spectrum Laboratories, Rancho Dominguez, CA), and tygon microbore tubing as described in Yamamoto and Pehek (1990). All junctions were glued with waterproof steel bond epoxy (Gorilla Glue) and allowed to cure before implantation.

Intracranial surgery occurred immediately after removal of the EtOH bottle on the last day of drinking (Day 28). Rats in all glutamate quantification experiments (including control groups) underwent intracranial surgery and microdialysis. Rats were anesthetized with a cocktail of ketamine (80 mg/kg) and xylazine (12 mg/kg) according to the IACUC protocol before undergoing stereotaxic surgery in the morning. Once fully anesthetized, the head was placed into a Kopf stereotaxic frame and the skull was exposed. According to Halpin *et al.* (2014), a hole was then drilled through the skull directly above the striatum [anteroposterior, +1.2; mediolateral (ML) ±3.0; dorsoventral (DV), -6.5 mm]. A microdialysis probe was slowly lowered into the striatum and secured to the skull with cranioplastic cement over three stainless steel screws. Buprenorphine (0.05 mg/kg) was administered subcutaneously via short action preparation and 1% lidocaine was applied topically to minimize pain immediately after the surgery according to the IACUC protocol. An additional injection of buprenorphine was administered 8-12 hrs after the surgery.

The following morning (day 29, 24 hrs after last day of EtOH or water drinking but prior to Meth or saline injections), artificial cerebrospinal fluid (aCSF) was infused with a Harvard Model 22 syringe infusion pump (Harvard Apparatus, Holliston, MA) at a flow rate of 1.5 μL/min through the microdialysis probe connected to a tether and single-channel swivel (Instech Laboratories, Plymouth Meeting, PA). After a 1.5 hr equilibration period, 3 baseline dialysate samples were collected, each 30 minutes apart. Directly after the third baseline collection, the first Meth or saline injection was administered. Dialysate samples were collected every 30 minutes throughout the binge saline or Meth regimen until 2 hrs after the last injection. Body temperatures were monitored continuously throughout the day. Probe placement was verified histologically after experiments.

Glutamate recovery (%) of the probes was tested *in vitro* in test tubes containing aCSF and glutamate at 37 ^{o}C. Perfusion flow rate was 1.5 μL/min and the difference between the known glutamate concentrations *in vitro* vs. the concentration of glutamate collected in the perfusate after 1 hr determined the relative recovery of glutamate for each probe.

Extracellular Glutamate

High-performance liquid chromatography (HPLC) with electrochemical detection was used to measure glutamate concentrations after *o*-pthaldialdehyde (OPA; Sigma cat #P0657) derivatization (Donzanti & Yamamoto 1988) in all rats that underwent microdialysis, including control groups. The stock derivatization reagent was made by dissolving 27 mg of OPA in 1 mL of 100% methanol and mixed with 9 mL of 0.1M sodium tetraborate (pH 9.4) followed by the addition of 5 μL β-mercaptoethanol (BME). This stock solution was then diluted 1:3 with 0.1 M sodium tetraborate buffer (pH 9.4) as described in Mark *et al.* (2007). Samples were loaded into an autosampler where 10 μL of the diluted stock derivatization reagent was

added to 20 µl of dialysate (or standard), mixed 3 times, and allowed to wait/react for 2 min before it was injected onto an OPA high-resolution column (150 x 4.6 mm; 5 µm particle size; Alltech). GLU was eluted using a mobile phase consisting of 0.1 M sodium phosphate and 0.1 mM EDTA in 10% methanol at pH 6.7. GLU was detected with an LC-4C amperometric detector (Bioanalytical Systems, Lafayette, IN) using a 6 mm glassy working electrode maintained at a potential of 0.6 V relative to an Ag/AgCl reference electrode. Glutamate content was analyzed via EZChrom ® software. Three baseline glutamate concentrations were averaged per rat to quantify basal glutamate in the striatum.

Dopamine Content in Striatum

Rats were rapidly decapitated without anesthetic 7 days after Meth and striata were dissected as described in Blaker and Yamamoto (2018). Briefly, tissue was sonicated in 0.25 N perchloric acid and centrifuged for 20 min at a speed of 14,000 x *g* at 4°C. Supernatant was collected and injected onto a C18 column (250 x 4.6 mm, 5 µm particle diameter, Varian) for HPLC using a LC-4C amperometric detector (Bio-analytical Systems). The mobile phase consisted of 21 g/L citric acid, 10.65 g/L $NaHPO_4$, 470 mg 1-octanesulfonic acid sodium salt (OSA), in 15% methanol at pH 4.0. Dopamine content was analyzed with EZChrom ® software. After complete removal of the supernatant, the remaining pellet was suspended in 1 N NaOH overnight at 4°C for protein quantification. The following day, protein content was determined via Bradford assay. Dopamine content (pg) from HPLC was normalized to total protein content (µg) per rat.

Statistical Analyses

Statistical analyses were performed using SigmaPlot V13.0 software for Windows (Systat Software). No exclusion criteria were pre-determined and therefore no animals were excluded from the study, i.e., the initial sample sizes are reflected in the total animals per group. Sample sizes are listed in Figure 1 as well as the results section. Samples sizes in all experiments were calculated via an a priori sample size calculation with G*Power for Windows 10 software (Faul *et al.* 2007). Differences in means previously reported from our lab (Blaker and Yamamoto, 2018; Mark *et al.* 2004) were used to determine p1 and p2 values. Specifically, the generic binomial test was chosen under the "exact" test family tab for differences between Meth alone and EtOH+Meth, and the a priori type of power analysis was selected. An alpha-value of 0.05, power of 0.80, p1 of 0.75 and p2 of 0.99 were used to calculate the sample sizes used throughout experiments. Blinding was not conducted. A one-way ANOVA was used to analyze the drinking behavior and t-tests were used to analyze glutamate transporters within the striatum, while the experiments including ceftriaxone were analyzed via two-way ANOVA within Meth-treated rats. Two-way repeated-measures ANOVAs were used to analyze ethanol intake over time in ± CEF groups (between-subjects) and extracellular glutamate during Meth. Post-hoc Tukey tests were used for multiple comparisons. All statistical analyses passed the Shapiro-Wilk normality test and Grubbs' outlier test (no data points were excluded). The cutoff for statistical significance was $\alpha = 0.05$ for all experiments.

Results

Rats voluntarily drink 10% EtOH over 28 days

EtOH (or water control) was quantified in grams consumed per 24 hrs and normalized to body weight (kg) per rat. Rats consumed 2.17 ± 0.35 g/kg on Day 1, 2.67 ± 0.33 g/kg on Day 7, 3.22 ± 0.41 g/kg on Day 14, and 3.73 ± 0.19 g/kg on Day 28. A repeated-measures

ANOVA showed that rats significantly increased EtOH intake over the 28 days ($F_{(1, 68)}$=9.47, p=0.004; n = 72; Fig. 2). Figure 2 contains EtOH drinking behavior for all rats regardless of intervention (or lack thereof).

EtOH drinking decreases GLAST but not GLT-1 in the striatum

GLAST and GLT-1 were quantified in the striatum 24 hrs after the last day of drinking. A decrease in GLAST ($t_{(1,8)}$=3.71, p=0.006) but not GLT-1 ($t_{(1,9)}$=0.18, p=0.86) was observed after EtOH compared to water (t-test, n = 4-7/group; Fig. 3A-B). Representative Western blots are shown in Fig. 3C.

CEF during EtOH drinking does not affect EtOH intake

To measure fluid intake between rats treated with CEF and those treated with saline vehicle (No CEF), bottles were weighed once per week in all groups (EtOH drinking with No CEF or CEF included in Figure 2). A repeated-measures 2-way ANOVA revealed a significant increase in EtOH over time in both CEF and No CEF groups but there were no differences between groups within each day (n = 24-49/group; Fig. 4A). Water intake did not change over time or between CEF and No CEF groups (Fig. 4B).

EtOH-induced decreases in GLAST are prevented by CEF

CEF (200 mg/kg) was administered to rats on the “EtOH off” days. 24 hrs after the last day of drinking, a 2-way ANOVA with Tukey post hoc analyses showed a significant interaction between EtOH and CEF ($F_{(1,49)}$ = 8.79, p<0.01) and that EtOH alone again decreased GLAST in the striatum compared to water groups (q=2.23, p=0.03). CEF treatment during

EtOH drinking protected against decreases in GLAST (Tukey post hoc, q=3.21, p=0.04 vs. EtOH+No CEF; n = 6-8/group; Fig. 5A). Representative Western blots are shown in Fig. 5B.

Decreases in GLAST from EtOH exposure leads to increased basal glutamate in the striatum 24h after the last day of drinking

Basal glutamate (μM) was quantified in the striatum 24 hrs after the last day of EtOH drinking by taking the mean of three baseline dialysate readings per rat and adjusting for average % glutamate recovery (21.3 ± 0.96%) of the probe. A 2-way ANOVA showed a significant interaction between EtOH and CEF ($F_{(1, 49)}$=9.87, p=0.003) and Tukey post hoc analyses revealed a significant increase in basal glutamate in rats that drank EtOH compared to rats that did not drink EtOH (EtOH+No CEF vs. Water+No CEF, q=8.86, p<0.001). CEF did not affect basal glutamate in water-only drinking rats (Water+CEF vs. Water+No CEF, p=0.78) but attenuated the increases in basal glutamate produced by EtOH drinking (EtOH+CEF vs. EtOH+No CEF; q=5.73, p<0.001; n = 12-16/group; Fig. 6).

Previous EtOH drinking enhances Meth-induced increases in extracellular glutamate in the striatum

A three-way ANOVA with EtOH, Meth, and time as factors revealed significant interactions between EtOH and Meth ($F_{(1, 437)}$=36.63, p<0.001) and between Meth and time ($F_{(18, 437)}$=37.38, p<0.001). Tukey post hoc analysis showed that Meth significantly increased extracellular glutamate (q=42.77, p<0.001 vs. saline control groups) and the increase was enhanced by prior EtOH drinking (EtOH+Meth vs. Water+Meth; q=12.83, p<0.001; n = 6-12/group; Fig. 7A).

There were no differences in EtOH vs. Water rats with regard to body temperature throughout Meth injections (average over time: 40.26 ± 0.61 ºC and 40.72 ± 0.44 ºC, respectively).

A subset of rats was treated with CEF during EtOH drinking, and CEF alone did not affect extracellular glutamate throughout the day of microdialysis (vs. Water+No CEF+Saline; data not shown). Analysis of cumulative glutamate concentrations via a two-way ANOVA within Meth-treated rats using EtOH and CEF as factors revealed a significant main effect of EtOH ($F_{(1,25)} = 5.26$, $p<0.05$) and a significant increase in Meth-treated rats that drank EtOH vs. Water ($q=4.20$, $p<0.01$). Tukey post hoc analyses showed that rats that received CEF during EtOH had comparable cumulative glutamate concentrations to the Meth alone group (represented as EtOH+CEF vs. Water+CEF) and CEF protected against the enhanced increases in glutamate (Tukey post hoc vs. EtOH+No CEF, $q=4.204$, $p<0.05$) (n = 6-10/group; Fig. 7B). CEF treatment did not affect Meth-induced hyperthermia compared to vehicle-treated (No CEF) controls (average over time: 39.98 ± 0.72 ºC and 40.21 ± 0.49 ºC, respectively).

Serial exposure to EtOH and Meth leads to caspase-3 mediated spectrin breakdown in the striatum 7 days after Meth

SBPs were measured in the striatum 7 days after Meth. Neither EtOH nor Meth alone increased caspase-3 mediated spectrin breakdown (data not shown), but a 2-way ANOVA with Tukey post hoc analyses within Meth-treated rats revealed a significant increase in SBPs at 120 kDa in rats that drank EtOH compared to Water drinking rats (i.e., EtOH+Meth+No CEF vs. Water+Meth+No CEF; $q=5.27$, $p=0.002$, Fig. 8A). CEF during EtOH (EtOH+CEF+Meth) blocked this effect ($q=4.72$, $p=0.03$ vs. EtOH++NoCEF+Meth; n = 6-10/group; Fig. 8A). A representative Western blot for all treatment groups is shown in Fig.

8B. A band at 145 kDa is also visible on the Western blot for SBPs, and this represents a SBP proteolyzed by a different protease known as calpain as described in Halpin et al. (2014). The calpain-mediated SBP bands were also analyzed and no differences were detected between groups (data not shown).

CEF administration during EtOH drinking attenuates enhanced dopamine depletions 7 days after Meth

Striatal dopamine concentrations were quantified at 7 days after Meth. A two-way ANOVA using EtOH and Meth as factors revealed that rats exposed to Meth displayed significantly less striatal dopamine (62.13 ± 3.80 pg/µg protein) compared to all saline controls after 7 days (including water and EtOH drinkers; 108.85 ± 2.91 pg/µg protein; data not shown; $F_{(1, 33)}=8.97$, p=0.03, n = 7-8/group). Within the Meth-treated rats, a 2-way ANOVA showed a significant interaction between EtOH and CEF (n = 6-10/group; $F_{(1, 27)}=9.78$, p=0.004). Tukey post hoc analyses revealed that Meth-treated rats previously exposed to EtOH displayed enhanced dopamine depletions compared to Water groups (i.e., EtOH+No CEF+Meth vs. Water+No CEF+Meth; q=8.73, p<0.001) and this was attenuated by CEF during EtOH (EtOH+No CEF+Meth vs. EtOH+CEF+Meth; q=5.34, p<0.001 vs. EtOH+No CEF within Meth groups; n = 6-10/group; Fig. 9).

Discussion

Intermittent access to EtOH over 28 days decreased GLAST immunoreactivity in the striatum and increased basal glutamate concentrations when measured 24 hrs after the last day of drinking. Prior EtOH drinking enhanced and synergized with the Meth-induced increases in extracellular glutamate within the striatum, spectrin proteolysis, and long-lasting depletions of dopamine. The β-lactam antibiotic CEF during the intermittent withdrawal days

from EtOH drinking protected against the decreases in GLAST and the exacerbated increases in extracellular glutamate produced by Meth. CEF also attenuated the breakdown of spectrin and the enhanced dopamine depletions 7 days after EtOH+Meth.

Rats increased EtOH intake over 28 days (Fig. 2) and despite consuming lower amounts than we have published (Blaker & Yamamoto, 2018), EtOH drinking resulted in decreased GLAST in the striatum at 24 hrs. In contrast, GLT-1 was unaffected by EtOH drinking (Fig. 3). The lack of changes in GLT-1 differs from the findings using alcohol-preferring rat strains (Rao & Sari 2012) however, others have reported no effect on GLT-1 after EtOH (Devaud 2001) despite a decrease in glutamate uptake (Melendez *et al.* 2005). These findings suggest other glutamate transporters (e.g. GLAST) are involved as evidenced by the current findings of a decrease in GLAST after voluntary EtOH drinking (Fig. 3). The mechanism by which EtOH drinking decreases GLAST is unknown, but could be due to inflammation. It has been shown that 28 days of intermittent access to EtOH increases pro-inflammatory cytokines as well as lipopolysaccharide (LPS) and COX-2 (Blaker & Yamamoto 2018). Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) decrease GLAST mRNA and protein, respectively (Dumont *et al.* 2014; Mandolesi *et al.* 2013) and could link EtOH drinking with decreases in GLAST.

The decreases in GLAST were blocked by CEF pre-treatment during EtOH drinking (Fig. 5) and could be mediated by the anti-inflammatory properties of CEF. Wei *et al.* (2012) showed that CEF blocks increases in TNF-α, IL-1β, and interferon-γ associated with traumatic brain injury in rats. Thus, EtOH-induced changes in glutamate neurotransmission may be closely associated with increases in inflammation. Regardless, the current findings provide new evidence for the ability of CEF to block decreases in GLAST after EtOH without altering GLAST under normal conditions in the water-drinking rats (Fig. 5). This is consistent with

previous studies reporting no change in GLAST or EAAC1 after CEF in naïve animals (Rothstein *et al.* 2005; Lee *et al.* 2008).

No changes in EtOH or water drinking were produced by CEF (Fig. 4A) and differ from the reports by Alhaddad *et al.* (2014) and Das *et al.* (2015). These differences could be due to rat strain differences as Sprague Dawley rats were used in the current study and not alcohol preferring rats (Alhaddad *et al.* 2014; Das *et al.* 2015). Additionally, we administered CEF during the intermittent "EtOH off" days and therefore, CEF was not present during actual EtOH drinking. Regardless, the effects of CEF on the supra-additive effects of Meth-induced increases in glutamate, spectrin proteolysis and decreases in dopamine content of EtOH exposed rats were not due to alterations in prior EtOH intake.

Glutamate transporter expression could be regulated by calcium-calmodulin-dependent protein kinase II (CaMKII) which specifically, targets GLAST/EAAT1 but not EAAT2 (Chawla *et al.* 2017). Autophosphorylation of CaMKII has recently been associated with alcohol dependence (Easton *et al.* 2013), and its autophosphorylation leads to phosphorylation of the N-methyl-D-aspartate (NMDA) receptor to promote its activation and contribute to excitotoxicity (Kitamura *et al.* 1993; McGlade-McCulloh *et al.* 1993). Similarly, CaMKII is altered after Meth application *in vitro* (Chen *et al.* 2016) and is required for Meth-induced increases in intracellular calcium (Goodwin *et al.* 2009).

The decrease in GLAST after the last day of EtOH drinking could be responsible for the increase in basal glutamate within the striatum of EtOH drinking compared to water drinking only rats (Fig. 6). To address this relationship, basal glutamate was quantified after CEF treatment during EtOH drinking. Glutamate concentrations in water drinking control groups

are comparable to other studies published in rodents (Griffin *et al.* 2015; Pati *et al.* 2016). Similar to the prevention of EtOH-induced decreases in GLAST, CEF also prevented the increases in basal glutamate after EtOH drinking (Fig. 6) and further supports the role of decreases in GLAST in mediating the increased basal levels of glutamate produced by EtOH. It remains to be determined if other glutamate transporters such as the cysteine-glutamate antiporter (xCT) located on astrocytes (Ottestad-Hansen *et al.* 2018; Pow 2001) are affected by EtOH drinking. The increases in glutamate observed in our study could be due to the combined effects of the reversed action of xCT to release glutamate from astrocytes (Ding *et al.* 2013; Lewerenz *et al.* 2013) in conjunction with the decrease in glutamate uptake by GLAST.

The increased glutamatergic state of EtOH-drinking rats was enhanced upon Meth exposure compared to Meth-treated rats that drank water only (Fig. 7). The finding that Meth alone acutely increased the extracellular concentrations of glutamate is consistent with previous studies (Nash & Yamamoto 1992). It should be noted that the increases in glutamate do not occur immediately after the 1st injection of Meth. This somewhat delayed increase may be due to the combined effects of release through polysynaptic mechanisms (Mark et al., 2004) and reduced uptake, the latter affected by prior EtOH exposure (Fig. 5) leading to a more rapid and enhanced accumulation of extracellular glutamate (Fig. 7A). Moreover, the enhanced increase in glutamate in rats exposed to EtOH+Meth was blocked by CEF during EtOH drinking. This evidence in conjunction with the finding that CEF blocked the decrease in GLAST observed after EtOH drinking supports the role of EtOH-induced GLAST changes in mediating the enhanced extracellular glutamate effects observed after the serial exposure to EtOH and Meth.

One potential limitation of these studies is the use of ketamine as an anesthetic. Ketamine is a NMDA receptor antagonist and may therefore affect glutamate signaling in the brain (Orser *et al.*, 1997). However, ketamine was administered during surgery after EtOH drinking had ended and before Meth injections began. Therefore, ketamine should be completely excreted from the system prior to any drug administration and should not affect Meth-induced excitotoxicity. Furthermore, all treatment groups underwent ketamine administration and intracranial surgery and therefore the proper controls were employed for any potential effects of ketamine alone.

The appearance of spectrin breakdown in EtOH+Meth rats (Fig. 8) is consistent with previous findings of excitotoxicity (Staszewski & Yamamoto 2006; Halpin *et al.* 2014; Tata & Yamamoto 2007) and can be explained by increases in extracellular glutamate resulting from decreased uptake and/or increased release (Figs. 5 and 7). Moreover, increases in glutamate are known to activate cysteine proteases such as calpain and caspase-3 (Wang *et al.* 1998) that cause proteolysis. More specifically, caspase-3 causes the proteolysis of the presynaptic cytoskeletal protein, spectrin localized to neurons (Goodman *et al.* 1995; Riederer *et al.* 1986). The proteolytic cleavage of spectrin results in breakdown products of different molecular weights and in the case of caspase-3 activation, causes the cleavage of spectrin into a 120 kDa breakdown product (Pike *et al.* 2001). The finding that the serial exposure to EtOH drinking and Meth-induced increases in the appearance of a 120 kDa spectrin breakdown product suggests caspase-3 mediated spectrin proteolysis as a consequence of the co-exposure to EtOH and Meth and a contributor to the synergistic effects of EtOH and Meth on striatal dopamine depletions (Blaker & Yamamoto 2018). Although we did not observe caspase-3-mediated proteolysis with either EtOH drinking alone or Meth alone, EtOH-induced neuron death and acute Meth-induced spectrin proteolysis have previously been observed (Warren *et al.* 2005; Warren *et al.* 2007; Staszewski & Yamamoto 2006; Halpin *et al.* 2014). One potential limitation of these

experiments is that spectrin breakdown products were quantified in tissue dissected from around the probe tract after intracranial surgery. However, all rats (controls included) received intracranial surgery and probe implantation, and therefore it is unlikely that the increase in spectrin breakdown produced by EtOH+Meth vs controls is due to damage from probe implantation in the striatum.

The synergistic depletions of dopamine content after the serial exposure to EtOH drinking and Meth injections can be explained by different mechanisms of glutamatergic activation. EtOH drinking alone decreased GLAST and increased the basal extracellular concentration of glutamate, presumably through the reduced uptake by astrocytes. GLT-1 was not affected by EtOH drinking and indicates a preference for degradation of astrocytic glutamate transporters rather than those on presynaptic neuron terminals. In contrast, current and previous studies showed Meth injections into rats naïve to EtOH cause acute increases in glutamate through polysynaptic mechanisms and the reversal of transporter (non-GLAST) activity (Mark *et al.* 2004; Halpin *et al.* 2014). Future studies are required, but these data suggest that the different mechanisms by which EtOH and Meth increase glutamate transmission can synergize to produce a supra-additive long-term depletion of dopamine content in the striatum that is attenuated but not blocked by the action of CEF on GLAST (Fig. 9).

Overall, the current study provides a novel role for decreased glutamate uptake induced by EtOH drinking to promote an enhanced neurotoxicity to the subsequent exposure to Meth and provides a new dimension to the co-morbidity associated with the frequent co-abuse of these drugs.

--Human subjects --

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods

ARRIVE guidelines have been followed:

Yes

=> if it is a Review or Editorial, skip complete sentence => if No, include a statement: "ARRIVE guidelines were not followed for the following reason:

"

(edit phrasing to form a complete sentence as necessary).

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Conflict of Interest

Bryan K. Yamamoto is a handling editor for the Journal of Neurochemistry. The authors do not declare any other conflicts of interest.

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Figure Legends

Fig. 1 *Timeline of experiments.* EtOH-drinking and water-control rats went through 2-bottle choice drinking paradigm for 28 days. Groups A and B did not receive any injections during the drinking paradigm while Groups C and D received saline or CEF injections on days without EtOH access. Groups A and C were euthanized 24 hrs after the last day of drinking (day 29) to measure GLAST and GLT-1 in the striatum. Groups B & D received probe implantation intracranially after the last EtOH exposure on Day 28, followed by Meth or saline injections on Day 29 during microdialysis to quantify extracellular glutamate. 7 days after Meth or saline, Groups B & D were euthanized and striata were dissected to measure spectrin breakdown products and dopamine content.

Fig. 2. *Drinking behavior in Sprague Dawley rats over 28 days.* Rats increased intake of 10% EtOH (g/kg/24 hrs) over time (*$p < 0.01$; $n = 72$). "n" indicates number of animals included. The thicker line in each box represents the mean of the group.

Fig. 3. *EtOH drinking decreases striatal GLAST but not GLT-1.* A) No changes were observed in GLT-1 immunoreactivity after EtOH compared to Water. B) GLAST immunoreactivity was decreased in the striatum 24 hrs after the last day of EtOH compared to Water (*$p < 0.01$). $n = 4$-7/group. "n" indicates number of animals included. C) Representative Western blots for GLAST, GLT-1, and the control β-actin are provided.

Fig. 4. *CEF during EtOH drinking does not affect EtOH intake nor water intake.* A) EtOH intake and B) water intake were not affected by CEF administration on the "EtOH off" days ($p > 0.05$). n = 24-49/group. The thicker line in each box represents the mean of the group.

Fig. 5. *Ceftriaxone during EtOH drinking blocks decreases in GLAST.* A) EtOH drinking decreased GLAST in the striatum at 24 hrs after the last day of EtOH drinking (*$p < 0.05$ vs. Water+No CEF) and this was blocked by CEF treatment during drinking (EtOH+CEF vs. EtOH+No CEF; $^{\#}p < 0.05$; n = 6-8/group). "n" indicates number of animals included. B) Representative Western blots for GLAST and β-actin are provided for all treatment groups.

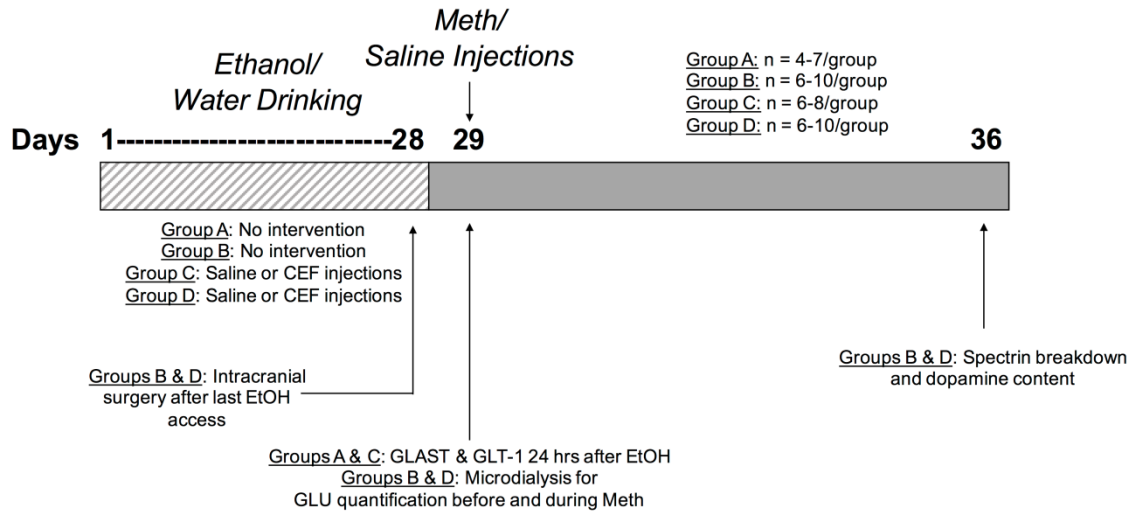
Fig. 6. *EtOH drinking increases basal glutamate in the striatum.* Extracellular glutamate was significantly increased in the striatum 24 hrs after the last day of EtOH (*$p < 0.001$ vs. Water+~~Saline~~No CEF). The EtOH-induced increases in glutamate were attenuated by CEF treatment during drinking ($^{\#}p < 0.001$ vs. EtOH+No CEF). n = 12-16/group. "n" indicates number of animals included.

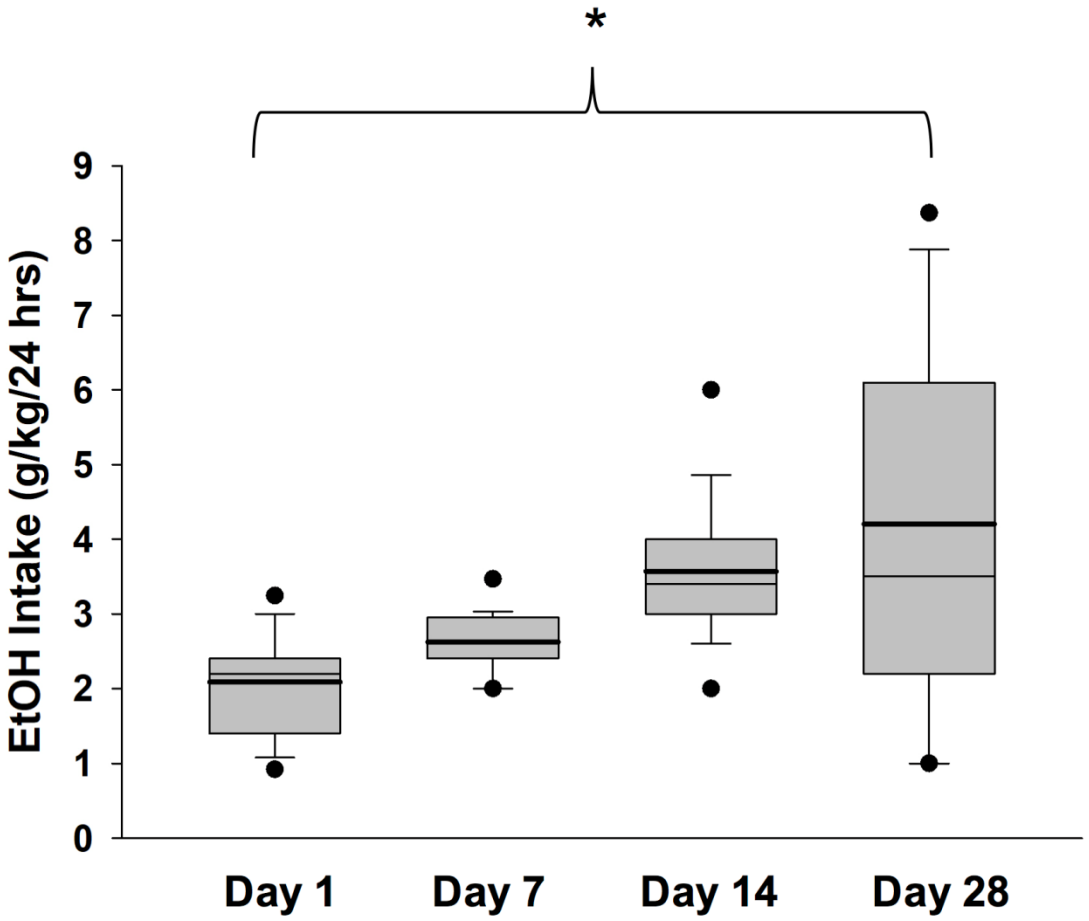
Fig. 7. *Previous EtOH exposure enhances Meth-induced increases in striatal glutamate.* A) Meth significantly increased glutamate in the striatum over the time course of four injections (*$p < 0.001$ vs. all saline vehicle groups) and this was further enhanced in rats previously exposed to EtOH (**$p < 0.001$ vs. Water+Meth; n = 6-12/group). Arrows denote injection time. B) Within Meth-treated rats, EtOH rats had significantly higher glutamate content (cumulative % increase compared to baseline) in the striatum compared to Water rats ($^{\&}p < 0.01$). This was attenuated by CEF administration during EtOH drinking ($^{\#}p < 0.05$ vs. EtOH+No CEF; n = 6-10/group). "n" indicates number of animals included. The dotted line

represents the ~~amount of~~ cumulative % change of glutamate from baseline in the striatum of Water+No CEF+Saline rats over time (2182.43 ± 22.98).

Fig. 8. *EtOH+Meth induce caspase-mediated spectrin proteolysis in the striatum 7 days after Meth.* Neither EtOH or Meth alone affected spectrin breakdown products (SBPs) 7 days after Meth, but within Meth-treated rats, EtOH+No CEF+Meth rats displayed increased SBPs in the striatum vs. Water+No CEF+Meth (*$p < 0.05$). EtOH+CEF prevented spectrin proteolysis ($^{\#}p < 0.05$; EtOH+CEF+Meth vs. EtOH+No CEF+Meth). n = 6-10/group. "n" indicates number of animals included. The dotted line represents the average Water+No CEF+Saline control group (100 ± 7.21% Water+No CEF+Saline).

Fig. 9. *CEF during EtOH drinking attenuates the enhanced dopamine depletions observed 7 days after Meth.* Meth significantly depleted dopamine in the striatum ($^{\&}p < 0.05$ vs. saline groups represented by gray bar). Dopamine was further depleted in Meth-treated rats previously exposed to EtOH (*$p < 0.001$ vs. Water+Meth groups) and CEF attenuated this synergistic effect ($^{\#}p < 0.001$ vs. EtOH+No CEF; n = 6-10/group). "n" indicates number of animals included. The gray bar represents the mean striatal dopamine content ± SEM of saline control rats (108.85 ± 2.92 pg/µg total protein).





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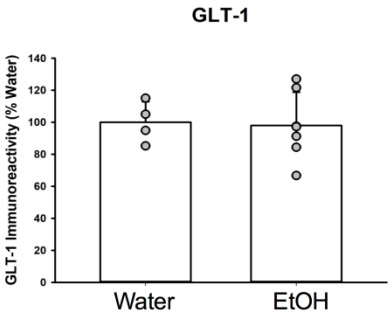

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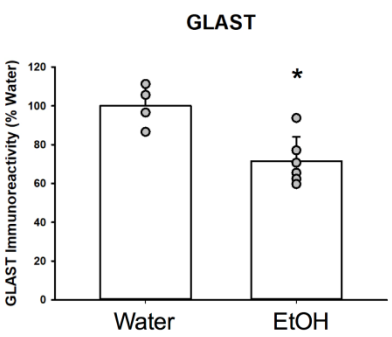

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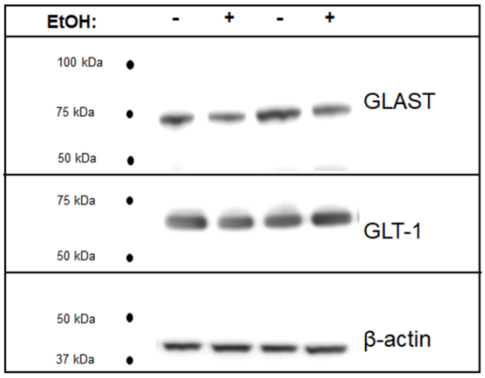

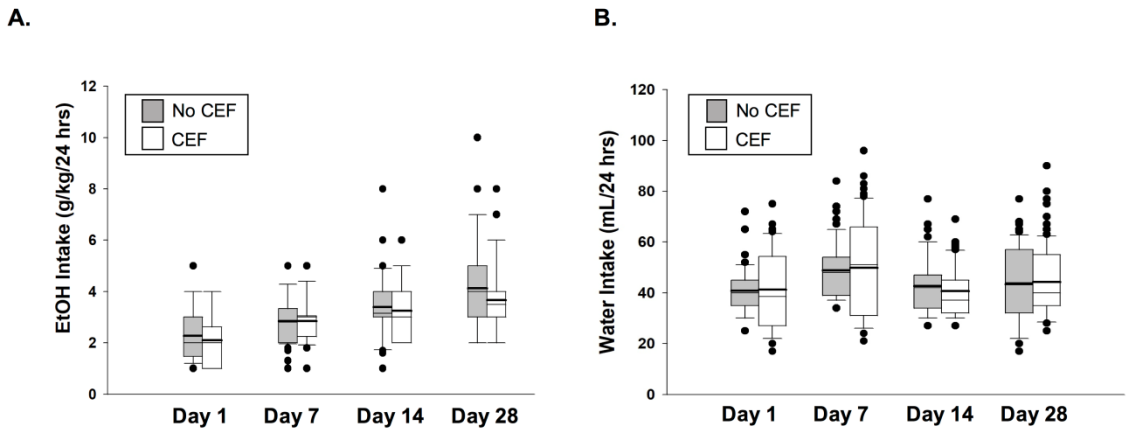

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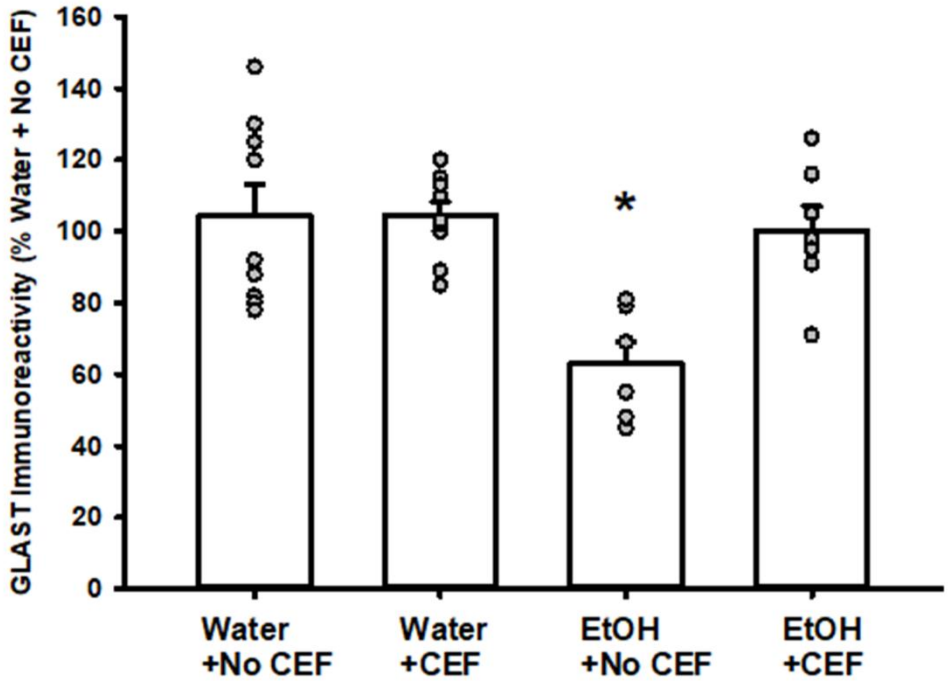

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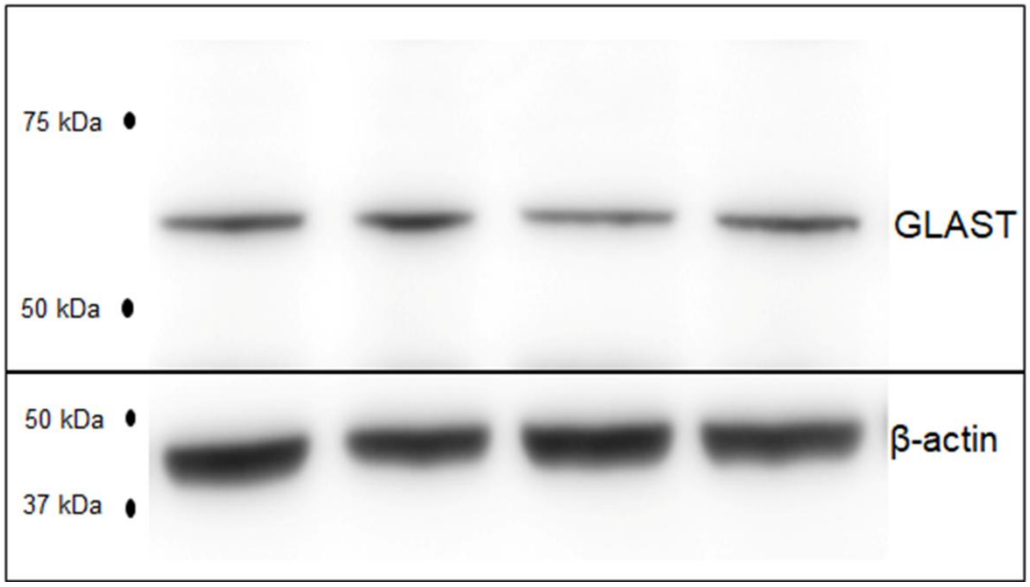

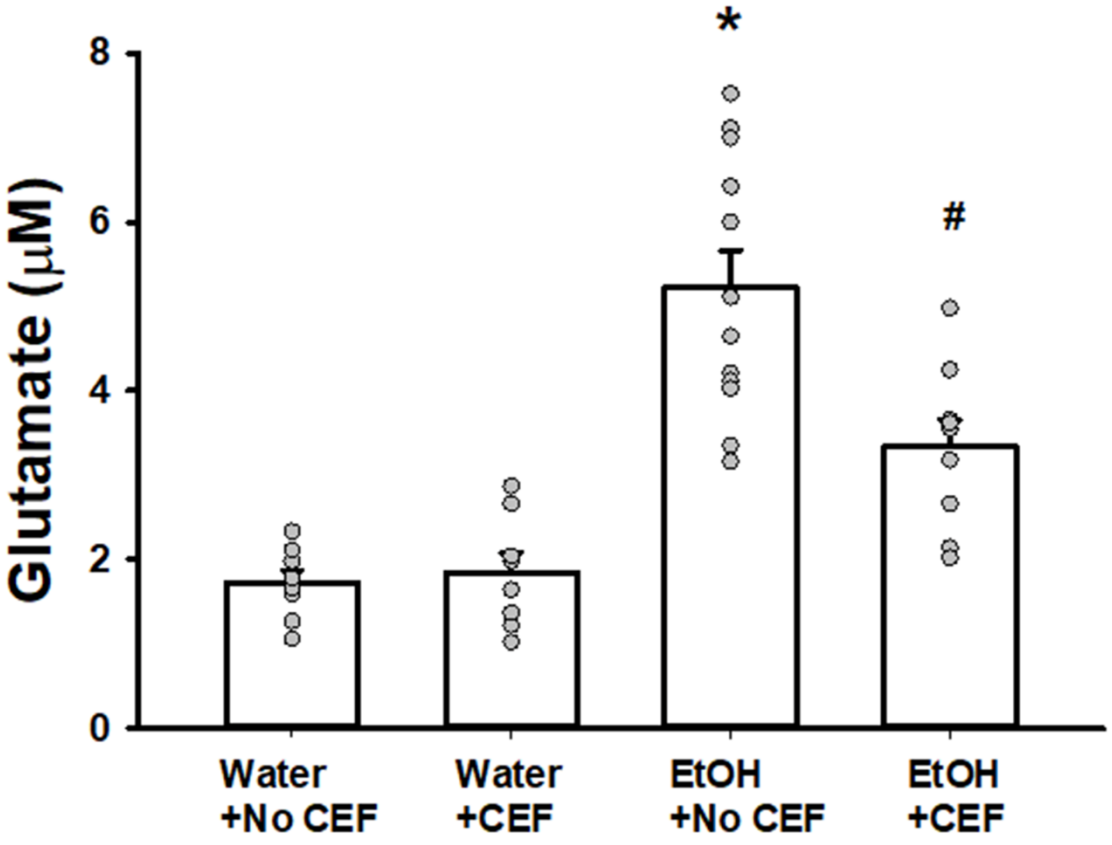

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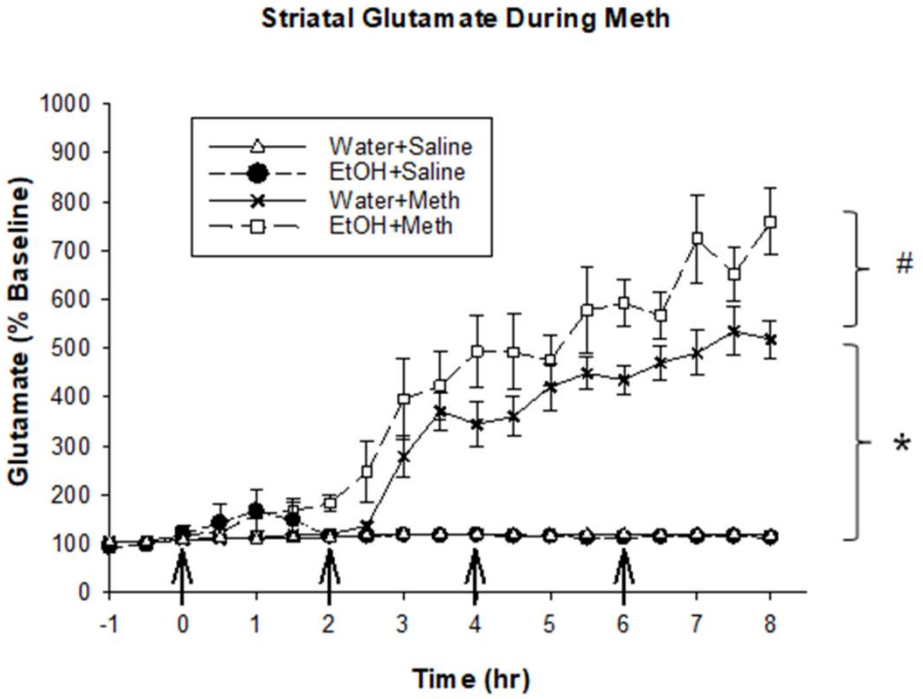

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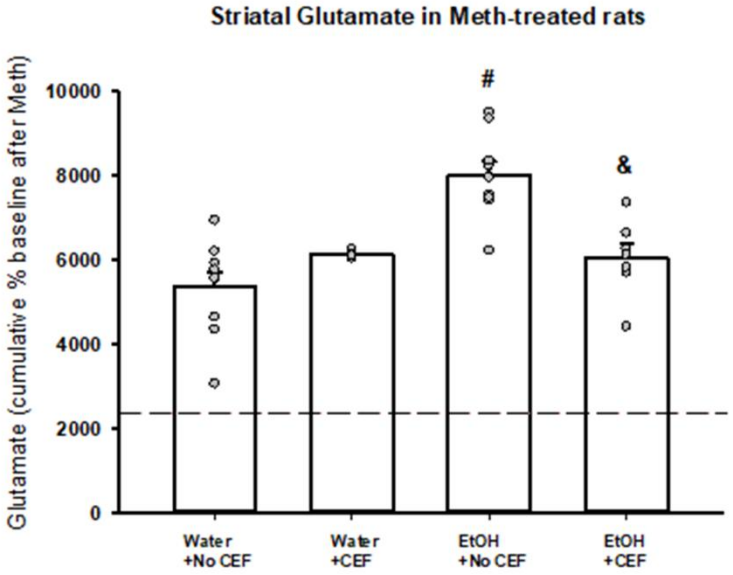

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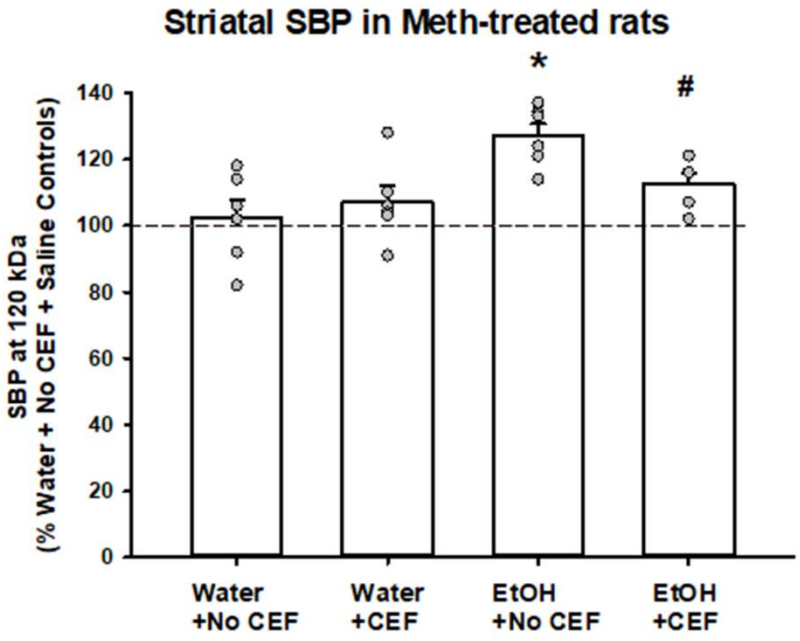

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